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Adriano E. Pereira
*Plant Genetics Research Unit, USDA/ARS*, adriano.pereira@ars.usda.gov

Thomas A. Coudron
*Biological Control of Insect Research Laboratory, USDA/ARS*, tom.coudron@ars.usda.gov

Kent Shelby
*Biological Control of Insect Research Laboratory, USDA/ARS*, Kent.Shelby@ars.usda.gov

B. Wade French
*North Central Agricultural Research Laboratory, USDA/ARS*

Elisa J. Bernklau
*Colorado State University*

*See next page for additional authors*

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Research



# Comparative Susceptibility of Western Corn Rootworm (Coleoptera: Chrysomelidae) Neonates to Selected Insecticides and Bt Proteins in the Presence and Absence of Feeding Stimulants

**Adriano E. Pereira,[1,5] Thomas A. Coudron,[2] Kent Shelby,[2] B. Wade French,[3] Elisa J. Bernklau,[4] Louis B. Bjostad,[4] and Bruce E. Hibbard[1]**

[1]Plant Genetics Research Unit, USDA/ARS, 205 Curtis Hall, Columbia, MO 65211, [2]Biological Control of Insect Research Laboratory, USDA/ARS, Columbia, MO, [3]North Central Agricultural Research Laboratory, USDA/ARS, Brookings, SD, [4]Department of Bioagricultural Sciences & Pest Management, Colorado State University, Fort Collins, CO, and [5]Corresponding author, e-mail: adriano.pereira@ars.usda.gov



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## Abstract

The susceptibility of western corn rootworm, *Diabrotica virgifera virgifera* LeConte, larvae to nine insecticides from five different classes and to Bt proteins eCry3.1Ab and mCry3A in the presence or absence of feeding stimulants, was estimated in filter paper and diet toxicity assays, respectively. The use of a synthetic feeding stimulant blend of the sugars glucose, sucrose, and fructose plus linoleic acid at a ratio of 30:4:4:0.3 mg/ml of distilled water was evaluated to determine whether they increase the efficacy of insecticides and Bt proteins. The efficacy of thiamethoxam diluted in solutions with feeding stimulants was significantly increased when compared to thiamethoxam dilutions in water (>60-fold). Differences in the efficacy of the other insecticide classes when diluted in feeding stimulant solutions were no greater than fivefold when compared to the insecticides diluted in water. The presence of corn root juice as a natural feeding stimulant diminished toxicity of the insecticides, except for thiamethoxam, even though larval fresh weight was higher when fed on root juice compared to feeding stimulant or water. The use of feeding stimulants in diet toxicity assays did not enhance efficacy of eCry3.1Ab nor mCry3A proteins. Feeding stimulants can be recommended in combination with thiamethoxam to increase larval mortality. These results are discussed in terms of applicability of feeding stimulants to improve susceptibility of western corn rootworm larvae to pesticides in general.

**Key words:** diet toxicity assay, feeding stimulant, sugar, $LC_{50}$, dose-response curve

The western corn rootworm, *Diabrotica virgifera virgifera* LeConte, is considered the most challenging corn (*Zea mays* L.) pest in the U.S. Corn Belt because of its ability to adapt to control tactics (Gray et al. 2009). Annual U.S. yield losses and control costs due to corn rootworm larval damage are estimated to exceed $2 billion (Wechsler and Smith 2018). Larval damage affects water and nutrient uptake by the corn roots (Riedell 1990, Hou et al. 1997). In heavy infestations, root system damage can lead to plant lodging, especially after heavy rain or wind gusts, causing additional yield losses from unharvested grain (Urías-Lopez and Meinke 2001).

Management options for western corn rootworm include the adoption of crop rotation using non-hosts such as soybean (*Glycine max* (L.)) (Branson and Ortman 1970, Branson and Krysan 1981, Clark and Hibbard 2004), corn hybrids expressing *Bacillus thuringiensis* Berliner (Bt) (Moellenbeck et al. 2001, Vaughn et al. 2005), and soil insecticides (Van Rozen and Ester 2010). However, resistance evolution by western corn rootworm to control tactics has been reported since the 1950s, first with resistance to synthetic cyclodiene insecticides (Ball and Weekman 1963) and subsequently to other insecticide classes (Meinke et al. 1998; Zhu et al. 2001; Pereira et al. 2015, 2017), crop rotation in eastern U.S. Corn Belt (Levine et al. 2002). The most recent case of resistance evolution is to Bt corn (Gassmann et al. 2011, 2014, 2016; Wangila et al. 2015; Jakka et al. 2016; Zukoff et al. 2016; Ludwick et al. 2017)

which currently is the primary control technique adopted by corn growers.

New technologies to manage corn rootworm are in constant demand. Recently, pyramided corn hybrids expressing more than one Bt toxin and/or more than one mode of action have been recommended by the Environmental Protection Agency (Head et al. 2017, Moar et al. 2017b) to delay corn rootworm resistance evolution and keep product longevity in the field (Roush and Shelton 1997, Roush 1998, Tabashnik and Gould 2012). Recent studies have also focused on toxins in bacteria species beyond *B. thuringiensis*, such as *Photorhabdus luminescens* (Bowen et al. 1998, Bowling et al. 2017), *Chromobacterium piscinae* (Sampson et al. 2017), *Pseudomonas chlororaphis* (Schellenberger et al. 2016, Tabashnik 2016), *Pseudomonas mosselii* (Wei et al. 2018), *Alkaligenes faecalis* (Moar et al. 2017a, Yalpani et al. 2017) and *Brevibacillus laterosporus* (Moar et al. 2017a). However, the need to augment and optimize efficacy of already registered products should also be considered under current scenarios.

Many western corn rootworm larval toxicity assays use artificial diet to evaluate insecticides, proteins and double-stranded RNA (dsRNA) are performed over 5- to 12-d periods; they are often limited due to microbial contamination during the course of the experiment (Siegfried et al. 2005, Magalhaes et al. 2007, Pereira et al. 2016, Meihls et al. 2018). Bacterial or fungal contamination of artificial diet during larval feeding bioassays has prevented reliable and accurate data collection and limited the duration of toxicity assays (Pereira et al. 2016). In addition, suboptimal nutritional factors in artificial diet can be a limitation for reliable bioassays (Deans et al. 2017). To address these problems, we have developed clean laboratory practices, larval handling techniques, effective egg sterilization, and an improved artificial diet formulation for western corn rootworm larvae which has enabled contamination free rearing for 10-d bioassays (Huynh et al. 2017, Ludwick et al. 2018, Meihls et al. 2018).

Given the time, labor, and high cost of insecticide discovery (Sparks 2013), development of more effective means to deliver pesticides to the larvae would be valuable environmentally and economically. Extensive research has been performed to understand western corn rootworm larval attractiveness to semiochemicals such as carbon dioxide (Strnad et al. 1986, Bernklau and Bjostad 1998) and benzoxazinoid secondary metabolites and/or their complexes with iron (Hibbard and Bjostad 1989, Bjostad and Hibbard 1992, Hu et al. 2018). Other studies have identified compounds from corn roots with feeding stimulant or arrestant effects on western corn rootworm neonates, such as fructose, sucrose, glucose, and free fatty acids oleic or linoleic acid (Bernklau and Bjostad 2005, 2008; Bernklau et al. 2011, 2018). The synthetic feeding stimulants could potentially increase insect susceptibility to insecticides by increasing contact and ingestion of toxins. Corn root contents or exudates diffusing through the soil serve as attractants to western corn rootworm larvae. Accordingly, root extracts may contain chemoattractants that can be can be used to formulate insecticide-treated baits. For example, a feeding stimulant mix of glucose, fructose, sucrose, and linoleic acid at proportion of 30:4:4:0.3 mg/ml was effective in promoting western corn rootworm larvae feeding (Bernklau and Bjostad 2008; Bernklau et al. 2011, 2018).

The objectives of the current research were to 1) determine baseline susceptibility of western corn rootworm neonates to nine different insecticides when diluted in water, feeding stimulant, or corn root juice, to 2) compare larval fresh weight after larvae feed on insecticides and stimulants and 3) to determine baseline susceptibility of western corn rootworm neonates to the Bt proteins eCry3.1Ab and mCry3A in the presence and absence of feeding stimulant on diet overlay toxicity assays.

## Materials and Methods

### Insects

Western corn rootworm eggs for insecticide bioassays were obtained from a non-diapause colony maintained at Plant Genetics Research Units, USDA/ARS, University of Missouri, in Columbia, MO. The colony was originally obtained from the USDA-ARS laboratory in Brookings, SD. For the diet toxicity assays with Bt proteins, non-diapausing western corn rootworm eggs were provided directly from the North Central Agricultural Research Laboratory, USDA/ARS, in Brookings, SD (mCry3A bioassays) and by Crop Characteristics, Inc., Farmington, MN (eCry3.1Ab bioassays). These colonies originate from the same background at USDA/ARS in Brookings, SD, and are susceptible to Bt proteins and insecticides.

### Insecticides, Feeding Stimulants and Bt Proteins

Technical grade (>98%) bifenthrin, tefluthrin, cyfluthrin, methyl parathion, tebupirimfos, DDT, carbaryl, and dimethoate were purchased from Chem Service (West Chester, PA). Thiamethoxam along with eCry3.1Ab and mCry3A proteins were provided by Syngenta Biotechnology (Research Triangle Park, NC). The Bt proteins eCry3.1Ab and mCry3A are delta-endotoxins that were modified from Cry3 protein (Walters et al. 2010). DDT was included in this study because DDT resistance can convey cross-resistance to pyrethroids, one of the few classes still used against corn rootworm adults and larvae (Pereira et al. 2017). With the exception of DDT, carbaryl, and methyl parathion, all of the insecticides are currently used for western corn rootworm control as adulticides (bifenthrin and dimethoate) or larvicides (tefluthrin, cyfluthrin, tebupirimfos, and thiamethoxam) (Ball 1981, Van Rozen and Ester 2010). Pyrethroids are typically divided into type I and type II, based on the presence (tefluthrin and cyfluthrin) or absence (bifenthrin) of an [alfa]-cyano group, respectively (Yu 2014). The pyrethroid type can lead to different susceptibilities, as reported in Pereira et al. (2017). In addition, cross-resistance studies with different insecticides are time saving and can point to the likely mechanism (i.e., target site insensitivity or enzyme activity-P450 monooxiganases) (Yu 2014) at a fraction of the cost of molecular studies such as RNA sequencing. The synthetic sugars glucose (Fischer Chemical, Fair Lawn, NJ), sucrose (MP Biomedicals, Inc., Solon, OH), fructose and the free fatty acid linoleic acid (Acros Organic, Thermo Fisher Scientific, Fair Lawn, NJ) were used at a ratio of 30:4:4:0.3 mg/ml, respectively, similar to Bernklau et al. (2011). We changed the linoleic acid proportion used in Bernklau et al. (2011) from 1.0 mg/ml as to 0.3 mg/ml in response to high mortality observed at or above 1.0 mg/ml. Corn root juice was used in this study as a natural feeding stimulant because it is known to elicit larval feeding (Bernklau and Bjostad 2005). Corn root juice was obtained by crushing roots from 3 to 4 d germinated pesticide-free corn seeds (Viking 60-01N variety, Albert Lea Seed, Albert Lea, MN) grown in 9-cm Petri dishes on distilled water moistened filter paper. The roots (3–4 roots, ~10-cm long) were cut from the seeds and, depending on the amount of root tissue, were pressed in 1.5 ml centrifuge tubes or in a garlic press. Juice was removed by using 100 µl micro syringe (Hamilton, Reno, NV) and used immediately.

### Insecticide Assays

Insecticide stock solutions were prepared in acetone (1 µg/µl); the amount of insecticide in acetone corresponding to the highest concentration tested of each insecticide was diluted in water, feeding stimulant, or corn root juice to make the 2× serial dilution similar to Magalhaes et al. (2007) and Pereira et al. (2015, 2017).

Six concentrations, plus control (feeding stimulants, root juice, or water only) were used to calculate the $LC_{50s}$. The concentrations were adjusted in each solution to yield between 0 and 100% mortality (determined in preliminary experiments). Concentrations used for each insecticide are detailed in Table 1. The tefluthrin concentration was calculated in µg/cm² because active ingredient formulation used in this research was diluted in acetonitrile, which is different from the powder formulation used in Pereira et al. (2015, 2017).

Bioassays were performed on 3.5-cm-diameter filter paper disks (Thermo Fisher Scientific, Waltham, MA) based on Bernklau and Bjostad (2005) and to avoid contamination. Diet toxicity assays are typically used with *B. thuringiensis* and dsRNA because mortality can take up to 4–7 d for *B. thuringiensis* (Siegfried et al. 2005) and up to 12 d for dsRNA (Pereira et al. 2016). One cohort of larvae was used in the bioassays with the three solutions (feeding stimulant, root juice, and water), with each insecticide concentration having three replicates similar as performed in Pereira et al. (2015, 2017). It is known that the larvae try to feed on the filter paper, but it is not clear if they actually ingest it (Bernklau and Bjostad 2005). Immediately after performing the 2× serial dilution by mixing and pipetting thoroughly, 100 µl of insecticide solution was applied onto the filter paper (Thermo Fisher Scientific, Waltham, MA) in each of the three replicates in 5.0-cm-diameter Petri dishes (Pall Corporation, Port Washington, NY; #7232 or 7242) and held for 10–20 min under a flow hood before transferring neonates. We used Pall Petri dishes because they seal completely prevention both evaporation of solution from the filter paper and larval escape (Magalhaes et al. 2007; Pereira et al. 2015, 2017). In preliminary tests, susceptibility and fresh weight of neonates exposed to insecticides diluted in a solution with acetone and applied to filter paper 24 h before delivery of the solutions was not different than the method used in this research (data not shown). Fifteen <36-h-old neonates were transferred to each filter paper in the Petri dishes 10–20 min after treatment. The completed Petri dishes were placed in a completely dark chamber at 24°C, 40–60% relative humidity Mortality was assessed after 24 h. Based on symptomology of insecticide intoxication by the western corn rootworm larvae (Pereira et al. 2015), neonates were considered dead if they were not moving, presented uncoordinated movement, or were unable to walk the length of their body. The insecticides used in this research act on contact, except thiamethoxam which is primarily effective when ingested (Tomizawa and Casida 2005; Yu 2014). After mortality was assessed, Petri dishes containing all larvae (dead and alive) were placed at –20°C for at least 24 h before recording larval fresh weight. Total fresh weight was recorded per each Petri dish in each concentration (total weight of larvae was divided by the number of larvae in each Petri dish to obtain single larval weight in mg) using a Sartorius Cubis ultra-micro scale (Sartorius Corporate, Göttingen, Germany; model MSU 6.6S-000-DM) to compare how much insecticide solution in feeding stimulant, corn root juice, or water the larvae ingested.

**Table 1.** Dose range (2× series dilution) of the insecticides in each respective solution used for the 24-h filter paper assay against western corn rootworm neonates

| Insecticides | Stimulant solution | Dose range |
|---|---|---|
| Bifenthrin | Feeding stimulant | 0.125–4.0 ng/cm² |
| | Root juice | 0.25–8.0 ng/cm² |
| | Water | 0.25–8.0 ng/cm² |
| Cyfluthrin | Feeding stimulant | 0.125–4.0 ng/cm² |
| | Root juice | 0.25–8.0 ng/cm² |
| | Water | 0.25–8.0 ng/cm² |
| Tefluthrin | Feeding stimulant | 0.04–1.28 µg/cm² |
| | Root juice | 0.08–2.56 µg/cm² |
| | Water | 0.08–2.56 µg/cm² |
| Methyl parathion | Feeding stimulant | 0.015625–0.5 ng/cm² |
| | Root juice | 0.03125–1.0 ng/cm² |
| | Water | 0.03125–1.0 ng/cm² |
| Dimethoate | Feeding stimulant | 3.25–120.0 ng/cm² |
| | Root juice | 7.5–240.0 ng/cm² |
| | Water | 7.5–240.0 ng/cm² |
| Tebupirimfos | Feeding stimulant | 0.015625–0.5 ng/cm² |
| | Root juice | 0.0390625–1.25 ng/cm² |
| | Water | 0.0625–2.0 ng/cm² |
| DDT | Feeding stimulant | 1.25–40.0 ng/cm² |
| | Root juice | 5.0–160.0 ng/cm² |
| | Water | 2.5–80.0 ng/cm² |
| Carbaryl | Feeding stimulant | 1.0–32.0 ng/cm² |
| | Root juice | 2.0–64.0 ng/cm² |
| | Water | 2.0–64.0 ng/cm² |
| Thiamethoxam | Feeding stimulant | 1.04–33.3 ng/cm² |
| | Root juice | 1.04–33.3 ng/cm² |
| | Water | 16.24–519.7 ng/cm² |

## Diet Toxicity Assays With *B. thuringiensis*

An improved western corn rootworm artificial diet (WCRMO-1, Huynh et al. 2017) was used for the toxicity assays. This artificial diet was improved from Pleau et al. (2002) by changing the proportion of some ingredients and adding wheat germ oil (Huynh et al. 2017). Sucrose is the only diet component as also present in the feeding stimulant. Therefore, by adding sucrose, glucose, fructose, and a fatty acid as a thin layer on top of the diet, it was expected that western corn rootworm larval feeding would increase, thus larvae would ingest more toxin. Egg sterilization was performed as in Ludwick et al. (2018). Two hundred microliters of the artificial diet were poured per well into 96-well plates (Costar # 3596, Corning Incorporated, Corning, NY) using a multi-channel dispenser pipette (Eppendorf, Hamburg, Germany; Eppendorf Repeater Plus/8 Pipette; # 022264109) and allowed to dry in a flow hood for about 10 min. Twenty microliters of eCry3.1Ab or mCry3A protein concentrations (0.0; 0.34; 1.03; 3.1; 9.29; and 27.87 µg/cm²) that cause between 0% and 100% mortality, diluted in buffer only (10 mM sodium carbonate/bicarbonate, pH 10), in sterile (autoclaved) water with feeding stimulant, or in buffer with feeding stimulant, were overlaid onto the surface of the diet by using an automatic multi-channel pipette (VWR International, Radnor, PA; # 89134-746) and allowed to dry in a laminar flow hood for about 1–1.5 h. One <24-h-old western corn rootworm neonate was transferred to plate. Plates were sealed using a pressure sensitive silicon adhesive 96-well cover (Excel Scientific, Inc., Victorville, CA; # TSS-RTQ-100) and one hole was punched per well using a # 1 insect pin. As in Ludwick et al. (2018), five replicates (plates) were used for each protein with each treatment (concentration) applied in one 12-well row per plate. A total of 60 neonates were used per treatment; mortality was recorded after 7 d. One batch of artificial diet and feeding stimulant was made and used within a 1-wk period.

Live larvae were removed from each concentration in the diet plates, placed in 1.5 ml micro centrifuge tubes containing 70% ethanol, and dried in an oven (Blue M Therm Dry Bacteriological Incubator, Model #602752) at 65°C for 48 h. Dry weight was measured with a Sartorius Cubis ultra-micro scale to generate the $EC_{50}$ (Siegfried et al. 2005).

### Data Analysis

For the insecticide bioassays, mortality data were obtained based on larval survival in each of the three replicates for each concentration of insecticide and each of the three solutions (feeding stimulant, root juice, and water) and averaged to obtain the mean and standard error. Only data from bioassays in which the natural background mortality, as per untreated control, was <20% were analyzed. $LC_{50}$ values of the insecticides tested were obtained by probit analysis (Finney 1971) using POLOPlus-PC software (LeOra Software 1987). Confidence intervals (95%) for susceptibility ratios between root juice or water and feeding stimulants in each insecticide were generated by POLOPlus-PC software as described in Robertson et al. (2007) and compared to test the significance of differences among the stimulants at the 95% level of confidence. In this test, if the 95% confidence interval estimated for a ratio does not contain 1.0 (i.e., 3.5–7.4), a significant difference exists between the $LC_{50}$ values compared (Robertson et al. 2007). Susceptibility ratios were calculated for each solution (water, feeding stimulants, or root juice) in each insecticide by dividing $LC_{50}$ of water or root juice by the $LC_{50}$ of feeding stimulants.

For diet toxicity assays, mortality data were analyzed using PROC PROBIT with Distribution=Logistic in SAS 9.4 (SAS Institute, Cary, NC) to generate $LC_{50}$ values. A nonlinear regression model using PROC NLIN was used to generate $EC_{50}$ values using SAS 9.4. Only replicates exhibiting control mortality <20% were considered in the analyses. Average larval weight was obtained as pooled larval weight/number of initial larvae. This calculation accounted for dead larvae (effectively giving them a weight of '0') as suggested in Siegfried et al. (2005).

## Results

### Insecticides

Thiamethoxam was the insecticide with the greatest increase in larval susceptibility in the presence of feeding stimulants, with >60-fold reduction in $LC_{50}$ when compared to water (Table 2). Corn root juice did not increase larval susceptibility in most insecticides, with $LC_{50}$s ranging from onefold (tefluthrin, dimethoate, tebupirimfos, and thiamethoxam) to 10-fold (DDT) increase when compared to feeding stimulants (Table 2). Except in methyl parathion (water), dimethoate (root juice), and thiamethoxam (root juice), all the root juice and water $LC_{50}$ values were statistically different from feeding stimulant $LC_{50}$, based on 95% confidence intervals of susceptibility ratios ($LC_{50}$s of root juice or water divided by $LC_{50}$ of feeding stimulant) that did not contain 1.0 (Table 2). Tefluthrin (root juice and water) and tebupirimfos (water) exhibited $LC_{50}$ values that were virtually the same when compared to feeding stimulant $LC_{50}$ (Table 2). Susceptibility of western corn rootworm neonates to most insecticides was higher when insecticides were tested with feeding stimulants compared to water or root juice (Table 2, Fig. 1). The susceptibility ratios between root juice and feeding stimulant ranged from 1.1-fold (dimethoate) to 9.7-fold (DDT), and between water and feeding stimulant ranged from 1.1-fold (methyl parathion) to 61-fold (thiamethoxam) (Table 2). Susceptibility of western corn rootworm neonates to tefluthrin and tebupirimfos was virtually the same in all solutions based on confidence intervals (Table 2, Fig. 1).

Based on confidence intervals, western corn rootworm neonates fed significantly more on insecticides diluted in root juice for controls and for the lower concentrations of insecticides when compared to water and feeding stimulant (Fig. 2). Additionally, this was true for DDT and methyl parathion in all concentrations based on confidence intervals (Fig. 2). Fresh weight of larvae that fed on insecticide diluted in root juice was similar among treatments in those higher concentrations for the insecticides bifenthrin and cyfluthrin, but was the highest for DDT and methyl parathion (Fig. 2). However, fresh weight was the lowest when diluted in root juice in the highest concentrations for the insecticides thiamethoxam, dimethoate, tefluthrin, and tebupirimfos (Fig. 2). Fresh weight of larvae that fed on water and feeding stimulant was similar in most insecticides in higher concentrations, with larvae that fed tebupirimfos, dimethoate, and thiamethoxam exhibiting the lowest fresh weight (Fig. 2).

### Bt Toxins in Diet Toxicity Assays

The addition of a sugar blend on the surface of the diet did not increase the efficacy of either Bt protein to western corn rootworm. Susceptibility of western corn rootworm neonates to Bt proteins with feeding stimulant diluted in sterile water or in buffer alone was not significantly different for either toxin based on overlapping confidence intervals (Table 3, Fig. 3). In contrast, $LC_{50}$ values of eCry3.1Ab + feeding stimulant diluted in buffer was 10-fold higher when compared to eCry3.1Ab + feeding stimulant diluted in sterile water or eCry3.1Ab + buffer only. For mCry3A assays, $LC_{50}$ values of mCry3A + feeding stimulant diluted in buffer was 75.3-fold and 41.6-fold higher when compared to mCry3A + buffer only or mCry3A + feeding stimulant diluted in sterile water, respectively (Table 3, Fig. 3). $EC_{50}$s were similar among treatments for both Bt proteins based on confidence intervals, with a trend of higher $EC_{50}$ values for *B. thuringiensis* mixed with feeding stimulant in buffer (Table 3), although the ratio was not higher than 3-fold.

Larval dry weight decreased as concentrations of both eCry3.1Ab and mCry3A increased (Fig. 4). Dry weight was higher in surviving larvae in control and the lowest at the two highest concentrations when fed without feeding stimulant for eCry3.1Ab (Fig. 4). For mCry3A, dry weight was similar within each concentration for all three treatments based on overlapping confidence intervals (Fig. 4).

## Discussion

In this study, we evaluated the susceptibility of western corn rootworm larvae to nine insecticides applied on filter paper and to Bt proteins eCry3.1Ab and mCry3A in diet toxicity assays, in the presence and absence of feeding stimulant (glucose, fructose, sucrose, and fatty acid at 30:4:4:0.3 mg/ml). Feeding stimulant substantially increased efficacy of thiamethoxam to western corn rootworm larvae when compared to all other insecticides (Table 2). This result indicates that thiamethoxam could potentially be used with feeding stimulant to improve western corn rootworm control. On the other hand, the presence of feeding stimulant mixed with Bt proteins overlaid on artificial diet did not increase susceptibility of western corn rootworm larvae (Table 3). All the tested insecticides have been used against western corn rootworm larvae or adults in the field, including tefluthrin, cyfluthrin and tebupirimfos as soil insecticides against larvae, thiamethoxam as seed treatment against larvae, and DDT, dimethoate, methyl parathion, carbaryl, and bifenthrin as adulticides (Ball 1981, Van Rozen and Ester 2010). In this study, feeding stimulant increased western corn rootworm neonate susceptibility to thiamethoxam by 61-fold when compared to water (Table 2). For the other insecticides, susceptibility of western corn rootworm larvae increased up to ~5.0-fold (cyfluthrin and dimethoate) compared to water, and up to ~10.0-fold (DDT) compared to root juice (Table 3). Interestingly, corn root juice lessened the effect of some of the insecticides (i.e., elevated the $LC_{50}$), except for tebupirimfos,

**Table 2.** Susceptibility of western corn rootworm, *Dibrotica virgifera virgifera* larvae to nine insecticides diluted in feeding stimulant (glucose, sucrose, fructose, fatty acid in a 30:4:4:0.3 mg/ml ratio), water, or corn root juice and applied to filter paper

| Insecticide/solution | $N^a$ | Slope (±SE) | $LC_{50}$ (95% CI) ng/cm$^2$ | $X^2$ (df) | Susceptibility ratio (95% CI)$^b$ |
|---|---|---|---|---|---|
| Bifenthrin | | | | | |
| Feeding stimulant | 315 | 2.03 (0.22) | 0.71 (0.42–1.13) | 8.36 (4) | - |
| Root juice | 360 | 6.07 (0.91) | 3.80 (3.30–4.34) | 5.51 (5) | 5.35 (4.21–6.80) |
| Water | 315 | 1.93 (0.20) | 1.67 (1.35–2.08) | 3.78 (4) | 2.35 (1.73–3.20) |
| Cyfluthrin | | | | | |
| Feeding stimulant | 315 | 2.30 (0.29) | 1.33 (0.97–1.83) | 4.01 (4) | - |
| Root juice | 315 | 2.70 (0.28) | 5.50 (3.79–8.49) | 8.52 (4) | 4.14 (3.13–4.47) |
| Water | 360 | 1.49 (0.17) | 6.52 (4.29–11.9) | 7.32 (5) | 4.90 (3.38–7.09) |
| Tefluthrin (µg/cm$^2$) | | | | | |
| Feeding stimulant | 315 | 3.38 (0.39) | 0.33 (0.25–0.43) | 5.18 (4) | - |
| Root juice | 315 | 5.04 (0.62) | 0.44 (0.29–0.69) | 2.91 (4) | 1.32 (1.07–1.63) |
| Water | 315 | 4.34 (0.48) | 0.41 (0.37–0.47) | 14.2 (4) | 1.25 (1.02–1.54) |
| Methyl parathion | | | | | |
| Feeding stimulant | 315 | 4.16 (0.57) | 0.08 (0.06–0.10) | 4.16 (4) | - |
| Root juice | 315 | 4.63 (0.79) | 0.39 (0.33–0.46) | 1.78 (4) | 4.83 (3.83–6.08) |
| Water | 315 | 5.62 (0.74) | 0.07 (0.06–0.08) | 0.06 (4) | 0.87 (0.71–1.07) |
| Dimethoate | | | | | |
| Feeding stimulant | 315 | 3.07 (0.32) | 19.2 (16.3–22.5) | 3.22 (4) | - |
| Root juice | 315 | 2.99 (0.33) | 20.7 (15.9–26.5) | 4.47 (4) | 1.08 (0.86–1.36) |
| Water | 405 | 3.85 (0.42) | 89.6 (58.1–140.2) | 25.9 (6) | 4.67 (3.76–5.80) |
| Tebupirimfos | | | | | |
| Feeding stimulant | 360 | 4.65 (0.54) | 0.11 (0.09–0.12) | 2.30 (5) | - |
| Root juice | 315 | 3.40 (0.49) | 0.15 (0.10–0.20) | 5.06 (4) | 1.43 (1.12–1.83) |
| Water | 360 | 4.85 (0.59) | 0.13 (0.12–0.15) | 1.75 (5) | 1.24 (1.04–1.49) |
| DDT | | | | | |
| Feeding stimulant | 315 | 2.24 (0.23) | 9.23 (6.43–13.7) | 6.69 (4) | - |
| Root juice | 270 | 3.40 (0.44) | 89.9 (60.3–162.1) | 6.34 (3) | 9.74 (7.52–12.61) |
| Water | 315 | 2.72 (0.27) | 20.7 (15.9–27.2) | 4.45 (4) | 2.24 (1.72–2.91) |
| Carbaryl | | | | | |
| Feeding stimulant | 315 | 3.93 (0.54) | 8.78 (6.56–11.2) | 4.73 (4) | - |
| Root juice | 315 | 1.30 (0.18) | 5.85 (2.94–9.38) | 5.19 (4) | 0.67 (0.46–0.96) |
| Water | 315 | 2.20 (0.23) | 19.2 (11.6–34.5) | 11.8 (4) | 2.19 (1.7–2.83) |
| Thiamethoxam | | | | | |
| Feeding stimulant | 315 | 1.20 (0.17) | 3.09 (2.07–4.22) | 2.97 (4) | - |
| Root juice | 315 | 1.98 (0.21) | 4.09 (2.68–05.95) | 6.29 (4) | 1.33 (0.88–1.99) |
| Water | 315 | 1.35 (0.18) | 188.5 (106.3–493.9) | 7.92 (4) | 61.1 (38.0–98.3) |

Mortality was recorded after 24 h.

[a]Number of insects bioassayed (three replicates with 15 larvae/plate for each insecticide concentration).

[b]$LC_{50}$ of water or root juice divided by $LC_{50}$ of feeding stimulant.

thiamethoxam, tefluthrin, and dimethoate, when comparing $LC_{50s}$. The $LC_{50}$ and $EC_{50}$ values for eCry3.1ab and mCry3A did not change significantly in the presence of feeding stimulant only (Table 3). However, the combination of feeding stimulant with 10 mM sodium carbonate/bicarbonate buffer reduced susceptibility of western corn rootworm to both Bt proteins, which could be an indicator of repellency or antagonism between the components (*B. thuringiensis*, sugars+fatty acid, and buffer).

It is known that insects exhibit different levels of susceptibility to insecticides within the same class, given their different molecular structure (Yu 2014). In the case of organophosphates, which are subdivided into six subclasses (Yu 2014), methyl parathion and tebupirimfos belong to the phosphorothioate class, and susceptibility ratios were similar between water and feeding stimulant (Table 2). However, bifenthrin and cyfluthrin, which are type I and II pyrethroids, respectively, had similar susceptibility ratios to all treatments (Table 2).

Baits containing attractants mixed with insecticides to kill western corn rootworm have been tested previously. Hibbard and Bjostad (1989) were the first to demonstrate that the use of semiochemicals from corn seedling volatiles increased the efficacy of four insecticides, including the pyrethroid tefluthrin, against western corn rootworm larvae. Siegfried et al. (2004) reported that the use of baits containing a feeding stimulant (cucurbitacin) with carbaryl promoted significant control of western corn rootworm beetles. Hibbard et al. (1995) documented that germinating corn extracts and 6-methoxybenzoxazolinone (MBOA) could be used to increase insecticide efficacy in the lab and field. In laboratory experiments, Bernklau and Bjostad (2008) isolated and identified a blend (glucose, sucrose, and fructose) plus fatty acid (oleic or linoleic acid) at a ratio of 30:4:4:0.3 mg/ml, respectively, as the primary feeding stimulants in corn root extracts. Carbon dioxide is attractive to western corn rootworm larvae (Strnad et al. 1986). Rodrigues et al. (2016) reported three receptor genes involved in $CO_2$ recognition by neonates based on comparison of gene expression among different western corn rootworm tissues and developmental stages.

Many insecticides are known to be repellent to a number of insect species (Zalucki and Furlong 2017), including western corn rootworm (Hibbard and Bjostad 1989). Repellent responses to the insecticides by western corn rootworm larvae could have played an

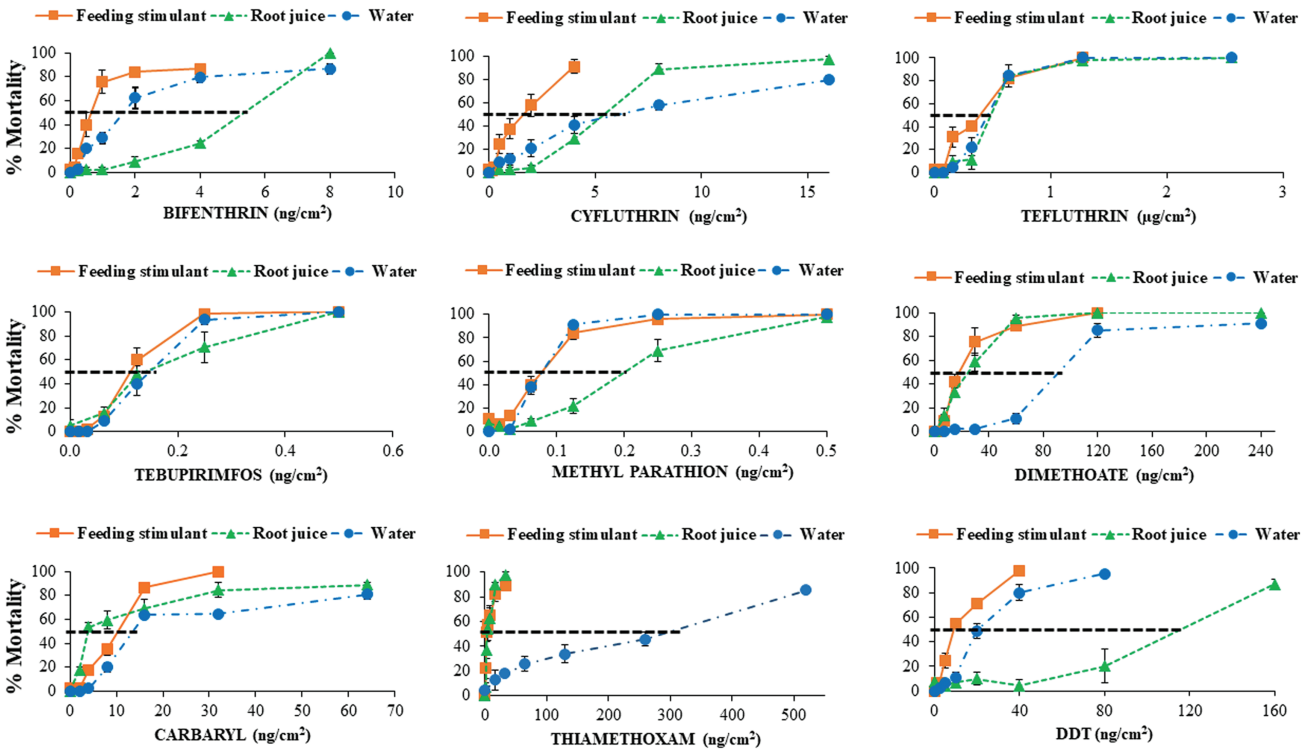


**Fig. 1.** Concentration mortality response curves for western corn rootworm neonates when contact-exposed to increasing insecticide concentrations of bifenthrin, tefluthrin, cyfluthrin, thiamethoxam, DDT, carbaryl, dimethoate, methyl parathion, and tebupirimfos diluted in feeding stimulant (square), corn root juice (triangle), or water (circle) in filter paper assays in small Petri dishes. Each mortality data point is the mean (±SE) of three replicates reported in 24 h bioassay. All insecticide concentrations are in ng/cm² except tefluthrin in (µg/cm²). Dashed lines indicate 50% mortality.

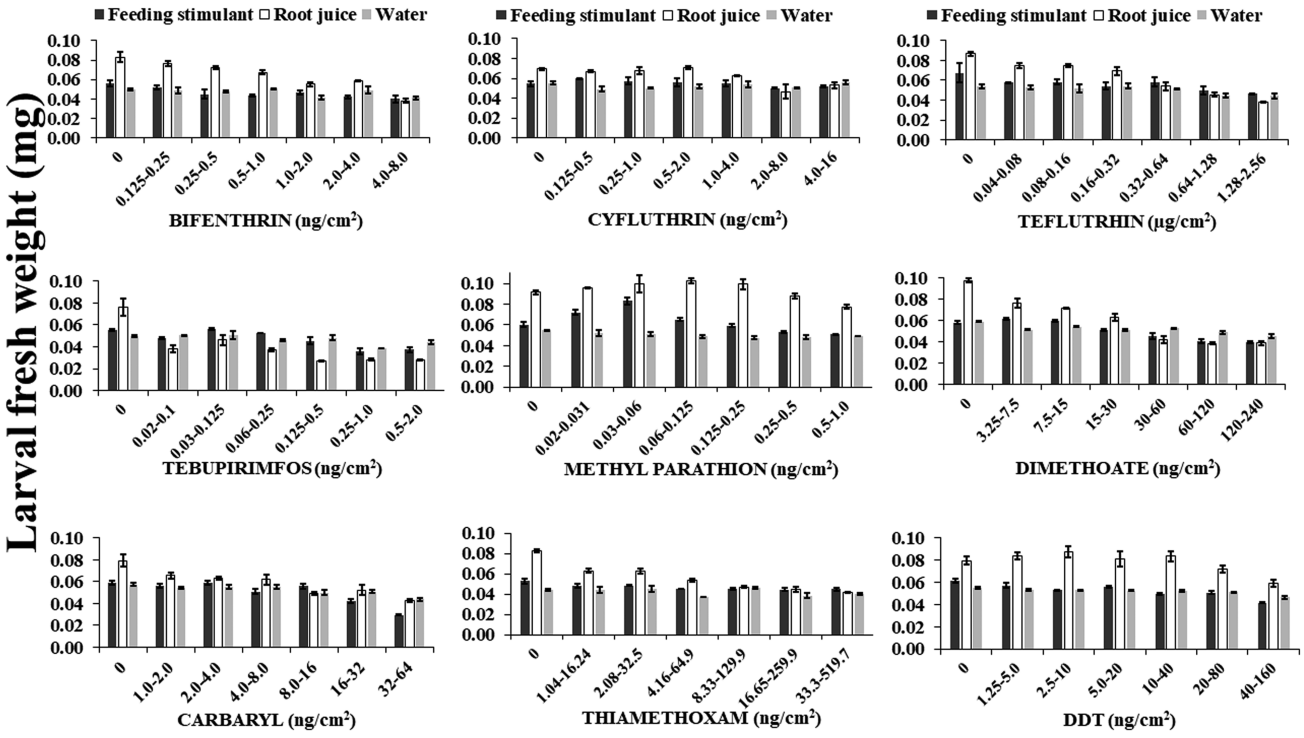


**Fig. 2.** Western corn rootworm larval fresh weight (mg) 24 h after exposure to the insecticides bifenthrin, cyfluthrin, tefluthrin, dimethoate, methyl parathion, tebupirimfos, thiamethoxam, carbaryl, and DDT, diluted in feeding stimulant, corn root juice, or water, applied onto filter paper disks. Each bar represents the mean (±SE) of three replicates with 15 larvae per replicate.

**Table 3.** Western corn rootworm, *Dibrotica virgifera virgifera* susceptibility to *Bacillus thuringiensis* proteins eCry3.1Ab and mCry3A in the presence and absence of feeding stimulant (glucose, sucrose, fructose, fatty acid in a 30:4:4:0.3 mg/ml ratio) in 7-d diet toxicity assays

| Bt protein | Treatments | $N^a$ | $LC_{50}$ (95% CI) µg/cm² | $EC_{50}$ (95% CI) µg/cm² |
|---|---|---|---|---|
| eCry3.1Ab + | Buffer only, no FS | 360 | 0.39 (0.16–0.80) | 0.47 (0.05–4.59) |
| | FS in sterile water | 360 | 0.37 (0.16–0.75) | 1.02 (0.15–7.04) |
| | FS in buffer | 360 | 3.52 (1.02–12.9) | 1.28 (0.53–2.95) |
| mCry3A + | Buffer only, no FS | 360 | 0.37 (0.15–0.79) | 0.35 (0.08–0.80) |
| | FS in sterile water | 360 | 0.67 (0.20–1.92) | 0.34 (0.10–0.63) |
| | FS in buffer | 360 | >27.87 | 0.80 (0.67–0.94) |

Lethal ($LC_{50}$) and effective concentrations ($EC_{50}$) (±95% confidence interval) were estimated from five replicates (diet plates) with 12 insects/treatment/replicate.
[a]Number of insects bioassayed.

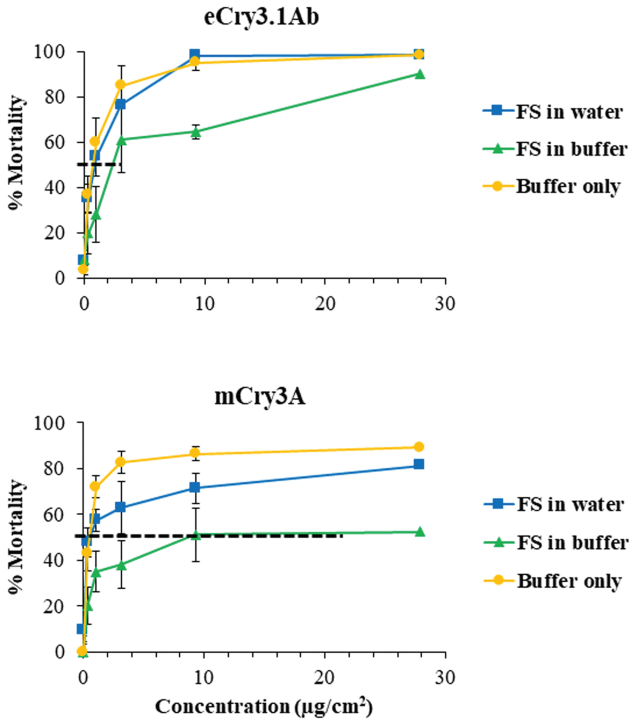


**Fig. 3.** Concentration mortality curves of eCry3.1Ab and mCry3A without and with feeding stimulant diluted in sterile water or buffer. Each data point is the mean (±SE) of 7-d mortality of 5 replicates, with 12 larvae per replicate for each concentration. Dashed lines indicate 50% mortality.

important role in the results of our study, even though we observed a drop in fresh weight in most of the insecticides for the treatments with feeding stimulant or root juice as doses increased (Fig. 2).

In our study, it was observed that thiamethoxam diluted in feeding stimulant was 61-fold more toxic to the larvae when compared to thiamethoxam in water. All the other insecticides act as better contact poisons for insects (Yu 2014),this may have affected western corn rootworm larval susceptibility results observed in this study. The increased susceptibility (lower $LC_{50s}$) of western corn rootworm neonates to the thiamethoxam when diluted in feeding stimulant suggests greater consumption by the larvae. Bernklau and Bjostad (2005) also reported a reduction in the amount of thiamethoxam required to kill western corn rootworm neonates when corn root extract was present compared to water only. Bernklau et al. (2011) observed a decrease of 300,000-fold in thiamethoxam $LC_{50}$ value to western corn rootworm neonates when diluted in glucose, sucrose, and fructose + linoleic acid at the proportion of 30:4:4:1 mg/ml. The difference in thiamethoxam susceptibility with feeding stimulant between this study and Bernklau et al. (2011) may be due to different insect colonies and to different time points used for assessing $LC_{50}$s. However, it is well known that susceptibility of same populations to a pesticide can change over the time due to natural variability, genetics, as well as unknown reasons (ffrench-Constant and Roush 1990; Robertson et al. 2007; Pereira et al. 2015, 2017). Regardless, $LC_{50}$s are just estimates and not constant values (Robertson et al. 2007). In our study, we checked mortality after 24 h compared to 4 h in Bernklau et al. (2011). When screening many western corn rootworm populations for resistance monitoring or baseline susceptibility, the use of simple and short time bioassays using filter paper in Petri dishes as performed in this study or as in Pereira et al. (2015, 2017) can be more appropriate.

Based on larval fresh weight (Fig. 2), it was clear that western corn rootworm neonates tried to feed consistently more on filter paper containing corn root juice than feeding stimulant or water. Additionally, larvae were observed feeding consistently more on root juice. However, susceptibility of neonates to some of the insecticides (bifenthrin, methyl parathion, and DDT) in root juice were lower compared to feeding stimulant. By crushing corn roots, it is expected that enzymes and other molecules from the tissues are released into the juice, and these may bind to, or degrade, the insecticides. Our results suggest that the degree of degradation or inactivation exceeded any gain that may have been realized by increased weight.

It was noticed that larval fresh weight was lower with root juice at higher concentrations in tefluthrin and tebupirimfos based on confidence intervals compared to the control and to the lower concentrations (Fig. 2). The lower weight may be a result of regurgitation and excretion observed at, or near, the time of death and thought to be caused by exposure to insecticides (data not shown). This phenomenon has also been reported in southern corn rootworm, *D. undecimpunctata howardi* Barber, although 3rd instar larvae were used in that study when topically exposed to the insecticides terbufos and tefluthrin (Michaelides and Wright 1997).

Using filter paper with insecticides diluted in distilled water, Pereira et al. (2017) reported low resistance ratios in western corn rootworm neonates whose parents showed high levels of resistance to DDT and tefluthrin compared to susceptible individuals. However, the use of feeding stimulant may assist in detecting levels of resistance in neonates after feeding on sugars which in turn could activate the enzymatic system in the body, including cytochrome-P450 monooxygenases, hydrolases, or glutathione-S-transferases, which are well-known enzymes involved in the resistance mechanisms (Yu 2014). Other studies have also reported low levels of resistance in young insect larvae/nymphs compared to older stages (Liu et al. 1995, Xi et al. 2015).

The addition of feeding stimulant in water did not result in a change in susceptibility of western corn rootworm to eCry3.1Ab or

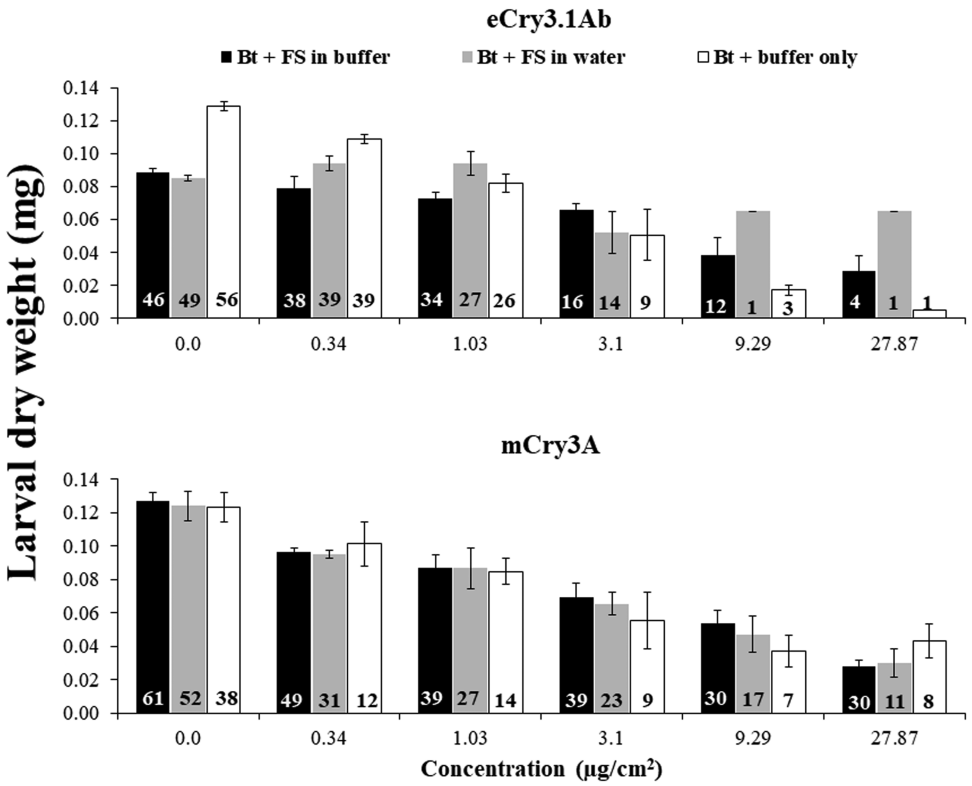


**Fig. 4.** Western corn rootworm larval dry weight (±SE) after exposure to different concentrations of eCry3.1Ab and mCry3A in 7-d diet toxicity assays. Bars contain the number of larvae that survived 7-d assays.

mCry3A proteins. In contrast, the addition of feeding stimulant in sodium buffer reduced western corn rootworm susceptibility to the proteins by 42-fold and 75-fold, respectively (Table 3). These results indicate that pH or solubility may be factors that affect susceptibility in toxicity assays. The differences for $EC_{50}$ values were not higher than threefold for both toxins and overlapped for all treatments in both proteins (except mCry + feeding stimulant in buffer), indicating that growth inhibition to either protein did not change substantially in the presence of feeding stimulant (Table 3). Bioassays with dsRNA mixed with feeding stimulant in diet should be evaluated given that dsRNA does not require addition of buffer for use in diet toxicity assays.

In summary, the results reported in this research show that the feeding stimulant tested in western corn rootworm larvae did not substantially increase insecticide efficacy in terms of $LC_{50}$s substantially, except for thiamethoxam, as previously reported in other studies (Bernklau and Bjostad 2005, Bernklau et al. 2011). Also, feeding stimulant did not increase susceptibility of western corn rootworm larvae to the Bt proteins eCry3.1Ab nor mCry3A in diet overlay toxicity assays but decreased susceptibility when mixed with 10 mM sodium carbonate/bicarbonate buffer at pH 10.0. Thiamethoxam is still used in seed treatments to control western corn rootworm larvae and should be considered as a potential candidate for incorporation with feeding stimulants to manage corn rootworm larvae. It is important to continue to search for other feeding stimulants or arrestants (Bernklau et al. 2018) that may be useful to improve corn rootworm larval control and help manage resistance evolution to other products including Bt corn hybrids.

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